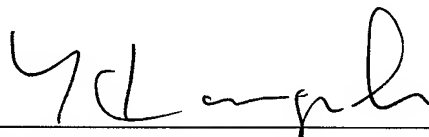


**VERIFICATION OF TRANSLATION**

Japanese Patent Application No. 2004-080454  
Filed on March 19, 2004

I, Yoshiyuki KAWAGUCHI, a citizen of Japan and the translator of the document attached, whose address is c/o SERA, TOYAMA, MATSUKURA & KAWAGUCHI, Acropolis 21 Building, 6<sup>th</sup> Floor, 4-10, Higashi Nihonbashi 3-chome, Chuo-Ku, Tokyo, Japan, state that the following is a true translation of the Japanese Patent Application No. 2004-080454 filed on March 19, 2004 to the best of my knowledge and belief.

Signed at Tokyo, Japan  
This 22nd day of January, 2008

A handwritten signature in black ink, appearing to read 'Yoshiyuki Kawaguchi', written over a horizontal line.

KAWAGUCHI, Yoshiyuki, Ph.D.  
Patent Attorney  
SERA, TOYAMA, MATSUKURA & KAWAGUCHI

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[Inventor]  
    [Address]        c/o JAPANESE FOUNDATION FOR CANCER RESEARCH  
                      HOSPITAL, 37-1, Kamiikebukuro 1-chome,  
                      Toshima-ku, Tokyo  
    [Name]            Kiyohiko HATAKE

[Inventor]  
    [Address]        c/o JAPANESE FOUNDATION FOR CANCER RESEARCH  
                      HOSPITAL, 37-1, Kamiikebukuro 1-chome,  
                      Toshima-ku, Tokyo  
    [Name]            Yasuhito TERUI

[Inventor]  
    [Address]        c/o Morinaga Milk Industry Co., Ltd.  
                      Nutritional Science Laboratory, 1-83,  
                      Higashihara 5-chome, Zama-shi, Kanagawa  
    [Name]            Akio YAMADA

[Inventor]  
    [Address]        c/o Morinaga Milk Industry Co., Ltd.  
                      Biological Science Laboratory, 1-83,  
                      Higashihara 5-chome, Zama-shi, Kanagawa  
    [Name]            Takuma SAKURAI

[Inventor]  
    [Address]        c/o Morinaga Milk Industry Co., Ltd.  
                      Biological Science Laboratory, 1-83,  
                      Higashihara 5-chome, Zama-shi, Kanagawa  
    [Name]            Yuji MISHIMA

[Inventor]  
    [Address]        c/o Morinaga Milk Industry Co., Ltd.  
                      Food Research & Development Laboratory,  
                      1-83, Higashihara 5-chome, Zama-shi,  
                      Kanagawa  
    [Name]            Takehito ITOH

[Applicant]  
    [I.D. Number]        000006127  
    [Name]              Morinaga Milk Industry Co., Ltd.

[Agent]  
    [I.D. Number]        100100549  
    [Patent Attorney]  
    [Name]              Yoshiyuki KAWAGUCHI

[Appointed Agent]  
    [I.D. Number]        100090516

[Patent Attorney]  
[Name] Hidemi MATSUKURA  
[Appointed Agent]  
[I.D. Number] 100089244  
[Patent Attorney]  
[Name] Tsutomu TOYAMA  
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[Name of Document] Claims 1 copy  
[Name of Document] Specification 1 copy  
[Name of Document] Abstract 1 copy

[Name of document] Claims

1. A drug for enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer, which comprises a lactoferrin hydrolysate that can be obtained by hydrolyzing lactoferrin with a hydrolytic enzyme and has an action of enhancing cytotoxic activity of the antibody drug in an antibody therapy of cancer as an active ingredient.

2. The drug according to claim 1, wherein the hydrolytic enzyme is pepsin.

3. The drug according to claim 1 or 2, wherein degradation rate of the lactoferrin hydrolysate is 6 to 20%.

4. The drug according to any one of claims 1 to 3, wherein the cancer is any one of breast cancer, B-cell lymphoma or colon cancer.

5. The drug according to any one of claims 1 to 4, wherein the cancer is a cancer having resistance to the antibody drug.

6. The drug according to any one of claims 1 to 5, wherein the action of enhancing cytotoxic activity of the antibody drug is an action of increasing sensibility of target cells to the antibody drug.

7. A drug for enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer, which comprises any one type or a mixture of two or more types of the following peptides of (a) to (d) as an active ingredient:

(a) a peptide having the amino acid sequence of SEQ ID NO: 2;

(b) a peptide having the amino acid sequence of the amino acid numbers 36 to 60 in the amino acid sequence of SEQ ID NO: 1, which includes substitution, deletion, addition or inversion of one or more amino acid residues,

and having an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer;

(c) a peptide having the amino acid sequence of SEQ ID NO: 3;

(d) a peptide having the amino acid sequence of the amino acid numbers 36 to 61 in the amino acid sequence of SEQ ID NO: 1, which includes substitution, deletion, addition or inversion of one or more amino acid residues, and having an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer.

8. The drug according to claim 7, wherein the cancer is any one of breast cancer, B-cell lymphoma or colon cancer.

9. The drug according to claim 7 or 8, wherein the cancer is a cancer having resistance to the antibody drug.

10. The drug according to any one of claims 7 to 9, wherein the action of enhancing cytotoxic activity of the antibody drug is an action of increasing sensibility of target cells to the antibody drug.

11. Food or drink comprising the drug according to any one of claims 1 to 10.

[Name of document] Description

[Title of invention] Drug for Cancer Therapy

[Technical field]

The present invention relates to a drug containing a lactoferrin hydrolysate mixture or a peptide of lactoferrin, which have an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer, as an active ingredient. More precisely, the present invention relates to a drug having an action of enhancing sensibility to an antibody drug of cancer cells having resistance to the antibody drug.

[Background Art]

The mechanism for antibodies to recognize a specific antigen and eliminate foreign substances out of organisms have long been studied. Immunoglobulin preparations are well known as application of antibodies to therapeutic treatments. However, they have problems such as insufficient titer and contamination of unknown infection sources.

The invention of the monoclonal antibody preparation technique by Milstein and Kohler brought breakthroughs in various antibody techniques. Today, in the so-called post-genome era, specific genes of tumor cells and pathogenic factors and expression products thereof are being successively elucidated. The antibody techniques are greatly contributing to development of research methods targeting them and therapeutic methods by utilizing them. Moreover, further advancement of the antibody techniques in future is being strongly expected and attracting attentions.

Antibody drugs targeting malignant tumor have strikingly advanced in the field of molecular target

therapy. Only two kinds of antibody drug products have been approved in Japan so far. However, currently known antibody drugs include at least those against malignant lymphoma (CD20, HLA-DR, CD5), acute myelogenous leukemia (CD33, CD66), chronic lymphocytic leukemia (CD52, CD20), acute lymphocytic leukemia (CD19, CD20, CD22), multiple myeloma (Id-idiotypic, HM1.24), breast cancer (HER2/neu), epithelioma (EGF-R, VEGF), colon cancer (17-1A, CEA), ovarian cancer (CA125) and so forth (for example, refer to Non-patent document 1).

As the action mechanisms of these antibody drugs against malignant tumor, roughly three kinds of mechanisms are considered. That is, the first are mechanisms in which, when antibodies bind to a surface of malignant tumor cell, certain signals are transmitted to the tumor cell to cause cell death. The second are mechanisms in which the effector cells (neutrophil, macrophage, NK cell etc.) existing in organisms recognize antibodies binding to tumor cells by antigen-antibody reactions. And they kill the tumor cells by the cytotoxic activity thereof. The third are mechanisms in which the complement components in organisms recognize antibodies binding to tumor cells by antigen-antibody reactions. And they induce cytotoxic action by a classical pathway among activation pathways of the complement system to kill the tumor cell (for example, refer to Non-patent document 2).

Many cancer patients could have been survived by the development of antibody drugs. However, reports have shown many cases where a treatment with an antibody drug had become difficult due to recurrence of malignant tumors acquiring resistance to the antibody drugs in the process of performing the treatments. Accordingly, combination therapies with a chemotherapeutic agents have been examined.

However, they have problems such as restriction of treatments for preventing adverse reactions (for example, refer to Non-patent document 3).

As a mechanism of acquisition of resistance to the an antibody drugs by tumor cells, many cases have been reported where expression of molecules called complement regulatory factors (CD46, CD55, CD59) increased on the surface of tumor cells. They inactivate the classical pathway of complements in the aforementioned third action mechanism, leading to the acquisition of the resistance (for example, refer to Non-patent document 4). It has been suggested that cytotoxic activity of the complement systems are degraded by such a mechanism, resulting in reduction of sensibility of target tumor cells to antibody drugs. Thus, development of a technique improving cytotoxic activity of the complement system against malignant tumor having resistance to an antibody drug has been desired.

Lactoferrin is a nonheme iron binding glycoprotein which exists in milk, saliva, tear, sperm, various mucus and so forth of mammals, and is a multifunctional protein having iron adsorption action, cell growth promoting action, immunoregulatory action and antimicrobial action. Bovine lactoferrin can be easily obtained in a large amount from raw skimmed milk or cheese whey routinely handled in dairy factories and can be readily utilized as a commercial product.

Lactoferricin (registered trade name of the applicant of the present invention) is a novel peptide isolated from an enzymatic hydrolysate of bovine lactoferrin by the inventors of the present invention for the first time (for example, refer to Patent document 1). Lactoferricin isolated from bovine lactoferrin has, for example, the amino acid sequence of SEQ ID NO: 2. Lactoferricin is an



extremely useful substance that exhibits an antimicrobial action at a low concentration on a wide range of microorganisms such as Gram-positive and Gram-negative bacteria and yeast that cause various diseases in humans and other animals. Most of other clinically used antimicrobial agents including antibiotics are chemical substances, which are foreign to human and animals. However, lactoferricin is a natural peptide that does not contain any chemical substance or chemically synthesized amino acid derivative, and therefore it is healthy and safe for human and animals. This is because lactoferricin is naturally produced in human stomach by decomposition of lactoferrin contained in usually ingested cow's milk by gastric pepsin. Therefore, lactoferricin can be utilized as a safe and effective antimicrobial agent in a wide variety of commercial products such as eye drops, buccals, cosmetics, skin lotions, therapeutic food and products for pets, and thus has an extremely great value.

[Patent document 1] Japanese Patent No. 2818056

[Non-patent document 1] Niizu Y. & Kato K., Saishin Igaku, Vol. 58, No.12, pp.5-12, 2003

[Non-patent document 2] Koyasu S. ed., Kinoshita T., Current Immunology Illustrated, pp.35-50, 2003

[Non-patent document 3] Hatake K., Blood Frontier, Vol. 12, No.11, pp.65-71, 2002

[Non-patent document 4] Oncogene, Vol. 22, pp.7359-7368, 2003

[Disclosure of the invention]

[Problem to be solved by the invention]

An object of the present invention is to provide a highly safe drug which causes no adverse reaction and has

an action of enhancing cytotoxic activity of the antibody drugs in the antibody therapies of cancer, in particular, drug-resistant cancer, food or drink containing the drug and a method for enhancing cytotoxic activity of the antibody drugs.

[Means to solve the problem]

The inventors of the present invention considered that, if we could enhance the sensibility of target tumor cells to the antibody drugs, the cytotoxic activity of the complement system should be sufficiently exhibited even if resistance to the antibody drug is confirmed in a patient with malignant tumor, and assiduously conducted research and development. As a result, they found that a lactoferrin hydrolysate mixture and a peptide of lactoferrin acted on target tumor cells to enhance sensibility of the cells to an antibody drug in an experimental system where a cytotoxic effect is caused by antibody and complement, and thus achieved the present invention.

The first invention of the present invention that achieves the aforementioned object is a drug for enhancing cytotoxic activity of the antibody drugs in the antibody therapies of cancer, which comprises a lactoferrin hydrolysate that can be obtained by hydrolyzing lactoferrin with a hydrolytic enzyme and has an action of enhancing cytotoxic activity of the antibody drug in an antibody therapy of cancer as an active ingredient.

The aforementioned first invention may be characterized by the following 1) to 5) as preferred embodiments.

- 1) The aforementioned hydrolytic enzyme is pepsin.
- 2) The degradation rate of the aforementioned lactoferrin hydrolysate is 6 to 20%.

- 3) The aforementioned cancer is any one of breast cancer, B-cell lymphoma and colon cancer.
- 4) The aforementioned cancer is a cancer having resistance to an antibody drug.
- 5) The aforementioned action of enhancing cytotoxic activity of an antibody drug is an action of increasing sensibility of target tumor cells to the antibody drug.

The second invention of the present invention that achieves the aforementioned object is a drug for enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer, which comprises any one type or a mixture of two or more types of the following peptides of (a) to (d) as an active ingredient:

- (a) a peptide having the amino acid sequence of SEQ ID NO: 2;
- (b) a peptide having the amino acid sequence of the amino acid numbers 36 to 60 in the amino acid sequence of SEQ ID NO: 1, which includes substitution, deletion, addition or inversion of one or more amino acid residues, and having an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer;
- (c) a peptide having the amino acid sequence of SEQ ID NO: 3;
- (d) a peptide having the amino acid sequence of the amino acid numbers 36 to 61 in the amino acid sequence of SEQ ID NO: 1, which includes substitution, deletion, addition or inversion of one or more amino acid residues, and having an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer.

The aforementioned second invention may be characterized by the following 6) to 8) as preferred embodiments.

- 6) The aforementioned cancer is any one of breast cancer,

B-cell lymphoma and colon cancer.

7) The aforementioned cancer is a cancer having resistance to an antibody drug.

8) The aforementioned action of enhancing cytotoxic activity of an antibody drug is an action of increasing sensibility of target tumor cells to the antibody drug.

Furthermore, the present invention provides food or drink comprising the drug of the aforementioned first or second invention.

[Advantageous effect of the invention]

The present invention relates to a drug for enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer, which comprises a lactoferrin hydrolysate mixture or a peptide of lactoferrin as an active ingredient, and target tumor cells can be efficiently killed or injured by enhancing cytotoxic activity of complement and antibody drug.

Furthermore, the drug of the present invention also has an effect of recovering cytotoxic activity of complement and antibody drug, in particular, against tumor cells having resistance to the antibody drug. Furthermore, the active ingredient of the drug of the present invention for therapeutic treatment of cancer having resistance can be obtained from a relatively inexpensive raw material such as cow's milk and therefore produced in a large scale.

Furthermore, the aforementioned active ingredient also exerts the same effect as that of the aforementioned drug when it is contained in food or drink, which is attached with an indication that the food or drink is for enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer.

[Best mode for carrying out the invention]

Hereafter, preferred embodiments of the present invention will be explained in detail. However, the present invention is not limited to the preferred embodiments described below and can be freely modified within the scope of the present invention. In the present specification, percentage is used on mass basis unless otherwise indicated.

The active ingredient of the drug for enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer or the drug for an antibody therapy of cancer of the present invention (henceforth also referred to simply as "the drug of the present invention") is a lactoferrin hydrolysate that can be obtained by hydrolyzing lactoferrin with a hydrolytic enzyme and has an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer (henceforth also referred to as "lactoferrin hydrolysate mixture") or any one type or a mixture of two or more types of the following peptides of (a) to (d) (hereinafter, also referred to as "partial peptide of lactoferrin"):

(a) a peptide having the amino acid sequence of SEQ ID NO: 2;

(b) a peptide having the amino acid sequence of the amino acid numbers 36 to 60 in the amino acid sequence of SEQ ID NO: 1, which includes substitution, deletion, addition or inversion of one or more amino acid residues, and having an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer;

(c) a peptide having the amino acid sequence of SEQ ID NO: 3;

(d) a peptide having the amino acid sequence of the amino acid numbers 36 to 61 in the amino acid sequence of SEQ ID NO: 1, which includes substitution, deletion, addition or

inversion of one or more amino acid residues, and having an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer.

SEQ ID NO: 2 corresponds to the amino acid numbers 36 to 60 in SEQ ID NO: 1. Furthermore, SEQ ID NO: 3 corresponds to the amino acid numbers 36 to 61 in SEQ ID NO: 1.

In the present invention, the term "antibody drug used for an antibody therapy of cancer" means a drug that contains an antibody binding to a surface of a cancer cell as an active ingredient and kills or injures the cancer cell by binding of the aforementioned antibody to the cancer cell. Actions of the antibody drug include inhibition of a cancer cell growth signal, killing or injuring cancer cells by activating a cell death signal, killing or injuring cancer cells by a complement-dependent cytotoxicity or antibody-dependent cellular-cytotoxicity, and so forth.

In the present invention, the expression "enhancing cytotoxic activity" encompasses, besides an effect of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer, that is, enhancing an action of a complement and/or an antibody drug to kill or injure target tumor cells, an action of increasing sensibility to an antibody drug of tumor cells having resistance to the antibody drug, that is, recovering cytotoxic activity of the complement and/or the antibody drug. Furthermore, the expression "having resistance to an antibody drug" means having resistance to an action of an antibody drug or complement to kill or injure tumor cells (cytotoxic activity). In other words, it means that the aforementioned action may be weak, or the tumor cells do not incur the action. Furthermore, resistance to an

antibody drug is not limited to resistance caused by a complement regulatory factor, and include resistance to an antibody drug caused by other factors.

A lactoferrin hydrolysate mixture and a peptide of lactoferrin, the active ingredient of the drug of the present invention, can be prepared by using lactoferrin as a starting material. As lactoferrin, there can be used commercially available lactoferrins and lactoferrins obtained from colostrum, transitional milk, nature milk or late lactation milk of mammals (for example, human, bovine and so forth) or processed products of these milks such as skimmed milk and whey as a raw material by isolating from the aforementioned raw materials with a conventional technique such as ion exchange chromatography. In particular, commercially available lactoferrin produced in an industrial scale (for example, one produced by Morinaga Milk Industry Co., Ltd.) can be preferably used. Furthermore, lactoferrins produced with microorganisms, animal cells, transgenic animals and so forth by using genetic engineering techniques can also be used.

Examples of the method for preparing a lactoferrin hydrolysate mixture, the active ingredient of the drug of the present invention, by using such lactoferrin include a method of hydrolyzing lactoferrin with a hydrolytic enzyme or the like in a conventional manner. As for specific method, the enzyme used for the hydrolysis of lactoferrin may be any hydrolytic enzyme so long as it is an enzyme that decomposes lactoferrin to produce peptides having an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer or a mixture thereof.

Examples of the hydrolytic enzyme include pepsin, pancreatine, trypsin, chymotrypsin, proteases derived from *Aspergillus oryzae* or *Streptomyces griseus* and so forth.

One or more types of hydrolytic enzymes may be used. When two or more types of enzymes are used, the enzymatic reactions may be performed simultaneously or separately. In the present invention, pepsin is particularly preferably used. Furthermore, it is also possible to use an endo-type protease in combination with an exo-type hydrolytic enzyme. The hydrolysis reaction time is preferably determined so that a preferred degradation rate can be attained while monitoring the degradation rate of the enzymatic reaction. To obtain a lactoferrin hydrolysate mixture, the active ingredient of the drug of the present invention, the degradation rate is preferably 6 to 20%, particularly preferably 8 to 12%.

The degradation rate of a protein can be calculated by, for example, measuring total nitrogen content of a sample by the Kjeldahl method (Ed. by The Japanese Society for Food Science and Technology, "Food Analysis Methods," p.102, Korin, 1984), measuring formol nitrogen content of the sample by the formol titration method (Ed. by Mitsuda et al., "Laboratory Manual of Food Engineering," First Volume, p. 547, Yokendo Co., Ltd., 1970) and using these measured values in the following equation.

$$\text{Degradation rate (\%)} = (\text{Formol nitrogen content} / \text{Total nitrogen content}) \times 100$$

When a preferred degradation rate is attained, the enzymatic reaction may be terminated. The enzymatic reaction can be terminated by inactivation of the enzyme in the hydrolysis solution, which can be carried out by a heat inactivation treatment in a conventional manner. The heating temperature and retention time of the heat inactivation treatment can be suitably selected as



conditions enabling sufficient inactivation taking into account thermal stability of the used enzyme, and, for example, the inactivation can be performed in the temperature range of 80 to 130°C for a retention time of 30 minutes to 2 seconds. The obtained reaction mixture is preferably adjusted to be within the pH range of 5.5 to 7 with an acid such as citric acid. When the lactoferrin hydrolysate mixture contains insoluble substances, it can be removed by either centrifugation or filtration.

The lactoferrin hydrolysate mixture obtained as described above can be utilized as an active ingredient of the drug of the present invention as it is, or can be lyophilized or spray-dried in a conventional manner and stored as powder. Furthermore, it can also be utilized for production of a lactoferrin partial peptide, another active ingredient of the drug of the present invention. Furthermore, the lactoferrin hydrolysate mixture may be obtained by adding a lactoferrin partial peptide to a hydrolysis product of lactoferrin obtained as above.

Specific examples of the aforementioned lactoferrin partial peptide include a peptide having the amino acid sequence of the amino acid numbers 36 to 60 in the amino acid sequence of SEQ ID NO: 1 shown in Sequence Listing and a peptide having the amino acid sequence of the amino acid numbers 36 to 61 in the amino acid sequence of SEQ ID NO: 1 shown in Sequence Listing (SEQ ID NO: 2). Hereinafter, the peptide having the amino acid sequence of the amino acid numbers 36 to 60 (SEQ ID NO: 3) is also referred to as lactoferrin F<sub>36</sub>-F<sub>60</sub>, and the aforementioned peptide having the amino acid sequence of the amino acid numbers 36 to 61 is also referred to as lactoferrin F<sub>36</sub>-A<sub>61</sub>.

When peptides comprising both the lactoferrin F<sub>36</sub>-F<sub>60</sub> and lactoferrin F<sub>36</sub>-A<sub>61</sub> are used as the active ingredients,

the mixing ratio of the lactoferrin F<sub>36</sub>-F<sub>60</sub> and lactoferrin F<sub>36</sub>-A<sub>61</sub> is preferably 1:20 to 20:1 (lactoferrin F<sub>36</sub>-F<sub>60</sub>:lactoferrin F<sub>36</sub>-A<sub>61</sub>), particularly preferably 1:10 to 2:3 (lactoferrin F<sub>36</sub>-F<sub>60</sub>:lactoferrin F<sub>36</sub>-A<sub>61</sub>).

The lactoferrin partial peptide, specifically the lactoferrin F<sub>36</sub>-F<sub>60</sub> and/or lactoferrin F<sub>36</sub>-A<sub>61</sub>, can be prepared, for example, according to the method for producing lactoferricin described in Japanese Patent Laid-open (Kokai) No. 5-238948 by using a lactoferrin hydrolysate mixture prepared as described above as a starting material. Further, the lactoferrin F<sub>36</sub>-F<sub>60</sub> and/or lactoferrin F<sub>36</sub>-A<sub>61</sub> may be any of a peptide prepared by a method of producing a synthetic peptide by chemical synthesis, a peptide produced by a method of producing it by synthesis of a recombinant peptide using a genetic engineering technique utilizing a gene recombination technique etc. and so forth, so long as it is a peptide having the amino acid sequence of the amino acid numbers 36 to 60 and/or the amino acid numbers 36 to 61 in the amino acid sequence of SEQ ID NO: 1. For example, as for the method of using a genetic engineering technique, the lactoferrin partial peptide can be obtained by preparing suitable primers on the basis of a nucleotide sequence encoding an amino acid sequence including a region of interest, amplifying the nucleotide sequence by PCR using the aforementioned primers and cDNA including the target nucleotide sequence as a template and expressing the obtained nucleotide sequence using a suitable expression system.

Furthermore, usual genes may contain a mutation such as substitution, deletion, insertion, addition or inversion of one or more nucleotides at one or more positions due to difference in species, genus, individuals and so forth, and

amino acid residues of a protein encoded by a gene having such a mutation may also have a mutation. The lactoferrin  $F_{36}-F_{60}$  and lactoferrin  $F_{36}-A_{61}$  that can be used for the present invention including such a mutation also fall within the scope of the present invention so long as the action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer having resistance is not degraded. The number of substituted, deleted, inserted, added or inverted amino acid residues that may be included in the lactoferrin  $F_{36}-F_{60}$  or lactoferrin  $F_{36}-A_{61}$  is preferably 1 to 12, more preferably 1 to 8, most preferably 1 to 5. Furthermore, the substitution of amino acid residues is preferably substitution of amino acids having similar property, i.e., so-called conservative substitution. If such a modified lactoferrin  $F_{36}-F_{60}$  or lactoferrin  $F_{36}-A_{61}$  is longer than the lactoferrin  $F_{36}-F_{60}$  or lactoferrin  $F_{36}-A_{61}$ , the added amino acid(s) is(are) preferably amino acid(s) corresponding to a sequence on the N-terminus side from the 36th position or on the C-terminus side from the 60th or 61st position of the lactoferrin. However, so long as the aforementioned modified lactoferrin  $F_{36}-F_{60}$  or lactoferrin  $F_{36}-A_{61}$  has a desired action, the added amino acid(s) may be an arbitrary amino acid(s).

The lactoferrin hydrolysate mixture or peptide of lactoferrin that can be used for the present invention has an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer, and the action can be measured according to a method using propidium iodide (PI, Dojindo, catalog No. P378) [Cytometry, Vol. 8, No. 4, pp.421-426, 1987] or a method using calcein-AM (Dojindo, catalog No. 341-07901) [Apoptosis, Vol. 3, No. 3, pp.195-202, 1998]. In the examples described later, the measurement method will be explained in detail.

As the drug of the present invention, a lactoferrin hydrolysate mixture or a peptide of lactoferrin can be orally or parenterally administered to a mammal including human in combination with a pharmaceutically acceptable carrier for pharmaceutical preparations. The dosage form of the drug of the present invention is not particularly limited, and can be suitably selected depending on the purpose of therapeutic treatment. Specific examples thereof include tablets, pills, powders, solutions, suspensions, emulsions, granules, capsules, syrups, suppositories, injections, ointments, patches, eye drops, nasal drops and so forth. In the production of the pharmaceutical preparation, additives commonly used for usual agents as pharmaceutical carriers such as excipients, binders, disintegrating agents, lubricants, stabilizers, flavoring agents, diluents, surfactants and solvents for injection can be used.

The amount of the lactoferrin hydrolysate mixture or peptide of lactoferrin contained in the drug of the present invention is not particularly limited and can be suitably selected. However, for example, the concentration of lactoferrin hydrolysate mixture contained in the pharmaceutical preparation may be 10  $\mu\text{g/ml}$  to 50  $\text{mg/ml}$ , preferably 50  $\mu\text{g/ml}$  to 10  $\text{mg/ml}$ , and the concentration of lactoferrin partial peptide in the preparation may be 0.5 to 200  $\mu\text{g/ml}$ , preferably 1 to 100  $\mu\text{g/ml}$ .

The administration time of the drug of the present invention is not particularly limited, and it can be administered before or after administration of an antibody drug or simultaneously with an antibody drug. The administration time can be suitably selected according to the method of therapeutic treatment for the objective cancer type. The administration method is suitably

selected also depending on the dosage form, age, sex and other conditions of patients, severity of symptoms of patients and so forth.

The dose of the active ingredient contained in the pharmaceutical preparation of the present invention is suitably selected depending on the administration method, age, sex and severity of disease of patients, other conditions and so forth. The dose of the lactoferrin hydrolysate mixture or lactoferrin partial peptide as the active ingredient is preferably in the range of 0.001 to 60 mg/kg/day, preferably 0.01 to 20 mg/kg/day, as a guideline, and the dose can be given once a day or at two or more times a day as divided portions.

The drug of the present invention is useful as a curative drug, a curative effect enhancing drug or a auxiliary curative drug in a therapeutic treatment using an antibody drug for a cancer, for example, any one of breast cancer, B cell lymphoma and colon cancer. The drugs of the present invention exhibit extremely marked efficacy against, in particular, cancer having resistance to an antibody drug among the aforementioned cancers. Examples of the antibody drug usable for the present invention include an anti-CD20 antibody (rituximab), anti-HER2 monoclonal antibody (trastuzumab), anti-17-1A (adhesion molecules of human epithelioma) antibody (edrecolomab) and so forth. In addition, antibody drugs usable in antibody therapy for cancer therapy can also be used. Moreover, not only the presently known antibody drugs, but also antibody drugs to be developed in future can also be used.

The drugs of the present invention are preferably used in antibody therapy together with an antibody drug (it is not limited whether they are simultaneously administered or separately administered), and it is also possible to use

them together with a known prophylactic or therapeutic drug (including an enhancing drug and auxiliary drug) for any of the aforementioned cancers in combination. The combined use can enhance the prophylactic and therapeutic effect for the aforementioned cancerous diseases, and can also reduce dose of the aforementioned prophylactic or therapeutic drug used in combination. Furthermore, the prophylactic or therapeutic drug for the aforementioned cancers to be used in combination may be contained in the compositions of the drugs of the present invention as an active ingredient, or it may not be contained in the compositions of the drugs of the present invention, but combined as a separate drug to constitute a commercial product and combined upon use.

The drug of the present invention or the lactoferrin hydrolysate mixture or lactoferrin partial peptide as active ingredient of the drug may be contained in food or drink. Form and property of the food or drink are not particularly limited so long as the effect of the active ingredient is not degraded, and they can be produced in a conventional manner by using raw materials usually used for food or drink except that the aforementioned active ingredient is added.

#### [Examples]

Hereafter, the present invention will be further specifically explained by referring to the following examples. However, the present invention is not limited to the following examples.

#### [Preparation Example 1]

##### Preparation of lactoferrin hydrolysate mixture

Bovine lactoferrin isolated from skim milk (Morinaga Milk Industry Co., Ltd., purity: about 90%) in an amount of

2.0 kg was dissolved in distilled water at a concentration of 5 mass %, and the solution was adjusted to pH 3.0 by addition of 1 N hydrochloric acid. Crystalline pepsin (Difco) was added at a ratio of 3 mass % of the substrate, and hydrolysis was allowed at 37°C for 4 hours. Then, the reaction mixture was heated at 80°C for 15 minutes to inactivate the pepsin, and 1 N sodium hydroxide was added to adjust the reaction mixture to pH 7.0. The insoluble substances were removed by filtration, and the filtrate was spray-dried to prepare about 1.9 kg of powdered lactoferrin hydrolysate. The decomposition rate of the produced lactoferrin hydrolysate mixture was 10%.

#### [Preparation Example 2]

##### Preparation of lactoferrin hydrolyzed peptide

A starting material prepared in the same manner as in Preparation Example 1 (lactoferrin hydrolysate mixture) in an amount of 600 g was dissolved in distilled water at a concentration of 5 mass %. About 3000 ml of a hydrophobic carrier (Butyl TOYOPERL 650 M, trade name, TOSOH CORP.) was equilibrated by sufficient washing with water. The solution of the starting material was mixed with the hydrophobic carrier in a tank provided with a stirrer, and then the solution was separated. The hydrophobic carrier was filled in a column (10 cm in length and 20 cm in diameter), and the aforementioned separated solution was loaded on the column. Then, the column was sufficiently washed by using water at a flow rate of 400 ml/minute until the absorbance of the washing solution at 280 nm became 0.06 or less.

Subsequently, 10 mmol hydrochloric acid was loaded on the column to elute lactoferrin hydrolyzed peptides, the eluate was mixed with an equal volume of McIlvaine buffer

(mixture of 177 parts by volume of 0.1 M acetic acid + 823 parts by volume of 0.2 M sodium primary phosphate, pH 7.0) and the peptides were adsorbed on the hydrophobic carrier. The hydrophobic carrier was filled in a column and washed with 6000 ml of the same buffer, and the lactoferrin hydrolyzed peptides were eluted with 9000 ml of McIlvaine buffer (mixture of 485 parts by volume of 0.1 M acetic acid + 515 parts by volume of 0.2 M sodium primary phosphate, pH 5.0). 10 mM hydrochloric acid and water was loaded on the column to regenerate the hydrophobic carrier, and pH of the eluate was adjusted to 7.0 with 1 N sodium hydroxide solution. The aforementioned eluate containing the lactoferrin hydrolyzed peptides was passed through this column, and the column was washed with 30 l of water to remove salts of the buffer. Then, 10 mM hydrochloric acid was loaded on the column to elute the lactoferrin hydrolyzed peptides, which were lyophilized to obtain about 10.5 g of powdered lactoferrin hydrolyzed peptides. Purity of the obtained lactoferrin hydrolyzed peptides was measured by HPLC, and as a result, it was found to be 99%. Furthermore, the amino acid sequences of the lactoferrin hydrolyzed peptides were determined, and as a result, it was confirmed that the produced lactoferrin hydrolyzed peptides contained both the peptides of lactoferrin F<sub>36</sub>-F<sub>60</sub> and lactoferrin F<sub>36</sub>-A<sub>61</sub>. The existence ratio of these peptides (lactoferrin F<sub>36</sub>-F<sub>60</sub>:lactoferrin F<sub>36</sub>-A<sub>61</sub>) was 1:6.

Hereafter, the present invention will be explained in detail with reference to the following test examples.

[Test Example 1]

This test was performed in order to examine the effect of addition of a lactoferrin hydrolysate mixture on cytotoxic action of complement and antibody drug.



#### (1) Test samples

Test Sample 1 was prepared with the lactoferrin hydrolysate mixture prepared in Preparation Example 1, of which concentration was adjusted to 1000 µg/ml in RPMI 1640 medium (Sigma, catalog number: R8758) containing 7.5% fetal bovine serum (Gibco, catalog number: 10099-141) and 1% penicillin-streptomycin (Gibco, catalog number: 15070-063) [henceforth this solution is referred to as "dilution solution"]. Furthermore, Control Sample 1 was prepared by diluting lactoferrin to a concentration of 1000 µg/ml with the dilution solution.

As target cells for measurement of cytotoxic action of complement and antibody drug, human Burkitt lymphoma Raji cells (ATCC CCL-86, obtained from American Type Culture Collection (10801 University Boulevard, Manassas, VA 20110-2209, USA)) were used, and the cells were suspended at a density of  $2 \times 10^6$  cells/ml by using the dilution solution to prepare a Raji cell suspension. As an antibody drug solution, rituximab (Zenyaku Kogyo, sold by Nippon Roche) that recognizes the CD20 antigen was used, and it was prepared at a concentration of 200 µg/ml using the dilution solution. As a complement solution, Human serum AB (Cosmobio, catalog number: 832000027) was used. These antibody drug solution and complement solution were also used for Test Example 2 to 4 described below.

Viability determination of the cells was performed by the method using propidium iodide (PI, Dojindo, catalog number: P378) [Cytometry, Vol. 8, No. 4, pp.421-426, 1987] or the method using Calcein-AM (Dojindo, catalog number: 341-07901) [Apoptosis, Vol. 3, No. 3, pp.195-202, 1998].

#### (2) Test method

The Raji cell suspension was added to each well of a

24-well culture plate (NUNC, catalog number: 143982) in a volume of 100  $\mu$ l each ( $2 \times 10^5$  cells each). The 24 wells were divided into four groups, to wells (6 wells) of one group among them, 50  $\mu$ l/well of the antibody drug solution and 250  $\mu$ l/well of dilution solution were added as "Addition of antibody drug". Further, to wells (6 wells) of another group, 100  $\mu$ l of the complement solution and 200  $\mu$ l of the dilution solution were added as "Addition of complement". Furthermore, to wells (6 wells) of another group, 50  $\mu$ l of the antibody drug solution, 100  $\mu$ l of the complement solution and 150  $\mu$ l of the dilution solution were added as "Addition of antibody drug + complement". To wells (6 wells) of the remaining one group, 300  $\mu$ l of the dilution solution was added as "No addition".

For the aforementioned four groups, 100  $\mu$ l/well of Test Sample 1 (4 groups  $\times$  2 wells), Control Sample 1 (4 groups  $\times$  2 wells) or the dilution solution (4 groups  $\times$  2 wells) mentioned above was added as "Test Sample Group 1", "Control Sample Group 1" and "Negative Sample Group 1", respectively.

By using the 24-well plate prepared as described above, the cells were cultured under the conditions of 37°C and 5% carbon dioxide (CO<sub>2</sub>) for 1 hour using an incubator (Napco, catalog number: 5300). Then, the cells of each sample group were collected, and the rate of dead cells in each was examined by the aforementioned method using PI. Cytotoxic activity was calculated based on a case where all the target cells were killed, which was taken as 100%.

### (3) Test results

The results of this test are as shown in Table 1. As clearly seen from the results shown in Table 1, with "Addition of antibody drug + complement", cytotoxic

activity was confirmed in each sample group ("Test Sample Group 1": 45.7%, "Control Sample Group 1": 24.3%, "Negative Sample Group 1": 30.7%), and it was found that, in particular, the lactoferrin hydrolysate mixture (Test Sample Group 1) markedly enhanced the cytotoxic activity (increase of about 50% compared with Negative Sample Group 1). On the other hand, the cytotoxic activity did not increase in Control Sample Group 1 compared with the negative sample group, and therefore it was found that the enhancement of cytotoxic activity was an activity specific to the lactoferrin hydrolysate mixture.

Table 1

|  | Cytotoxic activity (%) |                           |                            |
|--|------------------------|---------------------------|----------------------------|
|  | Test Sample<br>Group 1 | Control Sample<br>Group 1 | Negative<br>Sample Group 1 |
| Addition of<br>antibody drug<br>+ complement | 45.7                   | 24.3                      | 30.7                       |
| Addition of<br>antibody drug                 | 0.9                    | 1.0                       | 1.0                        |
| Addition of<br>complement                    | 1.5                    | 1.2                       | 1.1                        |
| No addition                                  | 1.2                    | 1.0                       | 1.3                        |

[Test Example 2]

This test was performed in order to examine change in sensibility of target tumor cells cultured with addition of the lactoferrin hydrolysate mixture during the culture for cytotoxic activity of complement and antibody drug.

(1) Test sample

The lactoferrin hydrolysate mixture prepared in Preparation Example 1 was diluted to a concentration of 1000 µg/ml with the dilution solution used in Test Example 1 to prepare Test Sample 2.

(2) Test method

The human Burkitt lymphoma Raji cells were suspended in 6.3 ml of the dilution solution with a cell number of  $2 \times 10^5$  cells/culture flask (BD, catalog number: 35-3014). As for the above suspension, a group to which 0.7 ml of Test Samples 2 mentioned above was added (Test Sample Group 2) and a group to which 0.7 ml of the dilution solution was added (Negative Sample Group 2) were prepared, respectively, and the cells were cultured for three days under the conditions of 37°C and 5% carbon dioxide (CO<sub>2</sub>) using an incubator.

The cells of both the sample groups were newly added with 10 ml each of the dilution solution and centrifuged (1100 rpm x 6 minutes) by using a centrifuge (KUBOTA, catalog number: 5910) to remove the supernatant. This procedure was repeated 3 times, and then the sensibility of the cells to the cytotoxic activity was examined by using the same systems as "Addition of antibody drug + complement" and "No addition" used in Test Example 1.

### (3) Test results

The result of this test are as shown in Table 2. As clearly seen from the results shown in Table 2, the sensibility to the cytotoxic activity provided by "complement + antibody drug" was clearly increased in the group of the target tumor cells cultured for 3 days with addition of the lactoferrin hydrolysate mixture (Test Sample Group 2) compared with Negative Sample Group 2, and thus it was found that the enhancing action clarified in Test Example 1 acted on the target tumor cells.

Table 2

|  | Cytotoxic activity (%) |                         |
|--|------------------------|-------------------------|
|  | Test Sample Group 2    | Negative Sample Group 2 |
| Addition of antibody drug + complement | 53.9                   | 34.8                    |
| No addition                            | 1.9                    | 1.5                     |

### [Test Example 3]

This test was performed in order to examine the effect of addition of a lactoferrin hydrolyzed peptide on the cytotoxicity of complement and antibody drug.

### (1) Test samples

The lactoferrin hydrolysate mixture prepared in Preparation Example 1 was diluted to a concentration of 1000 µg/ml with the dilution solution used in Test Example 1 to prepare Test Sample 3. Furthermore, the lactoferrin hydrolyzed peptide prepared by Preparation Example 2 was diluted to a concentration of 1000 µg/ml with the same dilution solution to prepare Test Sample 4.

## (2) Test method

The human Burkitt lymphoma Raji cells were suspended in the dilution solution at a density of  $2 \times 10^5$  cells/ml to prepare a cell suspension, and this cell suspension was added to each of 18 wells of a 96-well culture plate (NUNC, catalog number: 167008) in a volume of 50 µl each ( $1 \times 10^4$  cells each). The 18 wells were divided into two groups, to wells (9 wells) of one group, 10 µl/well of the antibody drug solution, 20 µl/well of the complement solution and 10 µl/well of the dilution solution were added as "Addition of antibody drug + complement". To wells (9 wells) of the remaining group, 40 µl of the dilution solution was added as "No addition". For each of the aforementioned 2 groups, 10 µl/well of Test Sample 3 (2 groups x 3 wells), Test Sample 4 (2 groups x 3 wells) or the dilution solution (2 groups x 3 wells) mentioned above was added as "Test Sample Group 3", "Control Sample Group 4" and "Negative Sample Group 2", respectively.

By using the 96-well plate prepared as described above, the cells were cultured under the conditions of 37°C and 5% carbon dioxide (CO<sub>2</sub>) for 1 hour using an incubator. Then, the cells of each sample group were collected, and the rate of dead cells in each was examined by the aforementioned method using PI. Cytotoxic activity was calculated based on a case where all the target cells were

killed, which was taken as 100%.

### (3) Test results

The results of this test are as shown in Table 3. As clearly seen from the results shown in Table 3, it was found that the lactoferrin hydrolyzed peptide (Test Sample Group 4) also markedly enhanced the cytotoxic activity exerted by complement and antibody (increase of about 67% compared with Negative Sample Group 2).

Table 3

|  | Cytotoxic activity (%) |                     |                         |
|--|------------------------|---------------------|-------------------------|
|  | Test Sample Group 3    | Test Sample Group 4 | Negative Sample Group 2 |
| Addition of antibody drug + complement | 77.4                   | 86.5                | 51.9                    |
| No addition                            | -0.1                   | 3.1                 | -0.5                    |

#### [Test Example 4]

In this test, cytotoxic activity enhancing action (recovery of sensibility) of the lactoferrin hydrolysate mixture was examined by using target tumor cells showing different susceptibilities for cytotoxic activity of complement and antibody.

#### (1) Test sample

The lactoferrin hydrolysate mixture prepared in Preparation Example 1 was diluted to a concentration of 1000 µg/ml with the dilution solution used in Test Example 1 to prepare Test Sample 5.

#### (2) Test method

Lymphoma-derived Daudi cells (ATCC CCL-213, obtained

from American Type Culture Collection), Raji cells (ATCC CCL-86, obtained from American Type Culture Collection), SKW6.4 cells (ATCC TIB-215, obtained from American Type Culture Collection) and lymphoma-derived ARH-77 cells (ATCC CRL-1621, obtained from American Type Culture Collection) having resistance to an antibody drug were each suspended in the dilution solution at a cell number of  $2 \times 10^6$  cells/ml to prepare cell suspensions, and each cell suspension was added to a 24-well culture plate (NUNC, catalog number: 143982) in a volume of 100  $\mu$ l/well ( $2 \times 10^5$  each).

Examination of the cytotoxic activity was performed in the same manner as that used for the "Addition of antibody drug + complement" system of Test Example 1. That is, to each of wells to which each cell suspension was added, 50  $\mu$ l of the antibody drug solution, 100  $\mu$ l of the complement solution and 150  $\mu$ l of the dilution solution were added, and 100  $\mu$ l of Test Sample 5 mentioned above or the dilution solution was further added as "Test Sample Group 5" and "Negative Sample Group 3", respectively.

By using the 24-well plate prepared as described above, the cells were cultured under the conditions of 37°C and 5% carbon dioxide (CO<sub>2</sub>) for 1 hour using an incubator (Napco, catalog number: 5300). Then, the cells of each sample group were collected, and the rate of dead cells in each was examined by the aforementioned method using PI. Cytotoxic activity was calculated based on a case where all the target cells were killed, which was taken as 100%.

### (3) Test results

The results of this test are as shown in Table 4. As clearly seen from the results shown in Table 4, decrease of the sensibility to the cytotoxic activity of complement and



antibody by the lactoferrin hydrolysate mixture was not confirmed for the Daudi cells, which show a high sensibility to the cytotoxic activity. For the Raji and SKW6.4 cells, the effect of enhancing the cytotoxic activity of complement and antibody was confirmed.

Furthermore, for the ARH-77 cells, which are lymphoma-derived cells showing resistance to the cytotoxic activity of complement and antibody (having resistance to an antibody drug), the effect of the lactoferrin hydrolysate mixture to eliminate the resistance to the cytotoxic activity was confirmed. It was considered that this was because the lactoferrin hydrolysate mixture had an effect of enhancing the sensibility to the cytotoxic activity for tumor cells having resistance to an antibody drug and recovered the cytotoxic activity of complement and antibody drug.

Table 4

|        | Cytotoxic activity (%) |                         |
|--------|------------------------|-------------------------|
|        | Test Sample Group 5    | Negative Sample Group 3 |
| Daudi  | 97.1                   | 96.4                    |
| Raji   | 49.0                   | 27.2                    |
| SKW6.4 | 38.9                   | 19.8                    |
| ARH-77 | 15.5                   | 0.0                     |

[Preparation Example 3]

Preparation of a drug for cancer therapy utilizing lactoferrin hydrolysate mixture as active ingredient

Lactose (Maigret, 600 g), maize starch (Nisshin Flour Milling Co., Ltd., 400 g), crystalline cellulose (Wako Pure Chemical Industries, 400 g) and the lactoferrin hydrolysate mixture produced in Preparation Example 1 (600 g) were sifted through a 50-mesh sieve (Yamato Scientific), placed in a polyethylene bag having a thickness of 0.5 mm, mixed

by inversion and filled in capsules (Japan Elanco, gelatin capsule No. 1, Op. Yellow No. 6 Body, empty weight: 75 mg) in an amount of 275 mg/capsule by using a full automatic capsule filling machine (Cesere Pedini, press type) to obtain 7000 capsules of the drug having an effect of enhancing cytotoxic activity of antibody drug.

[Preparation Example 4]

Preparation of a drug for cancer therapy utilizing lactoferrin hydrolyzed peptide as active ingredient

Crystalline cellulose (Wako Pure Chemical Industries, 20 g) was placed in a 1000-ml volume mortar (Nakashima Mfg. Co., Ltd.), added with 20 ml of water and mixed. Then, to the mixture, 25 g of lactose (Maigret) sifted beforehand through a 48-mesh sieve (Wakamori) was added, and 55 g of the lactoferrin hydrolyzed peptide prepared in Preparation Example 2 was added and mixed. The obtained wet mass was placed on a 20-mesh sieve (Wakamori) and manually extruded onto a stainless steel plate for drying to prepare granules, and the granules were quickly and uniformly distributed, placed in a drier and dried at 25°C for two days to obtain fine granules. These granules were sifted through a 20-mesh polyethylene sieve (Wakamori), and the granules passed through the sieve were spread on a large paper sheet, added with 2 g of magnesium stearate (Kanto Kagaku) sifted through a 48-mesh sieve beforehand and made uniform by manual mixing. The granules were made into tablets in a tablet making machine (Kimura Seisakusho, Model KT-2) using an R pestle having a diameter of 8 mm with setting compression pressure for the conditions of a tablet making number of 10, tablet weight of 6.2 g and Monsanto hardness of 3.5 to 5.0 kg to obtain 16 tablets of the drug having an effect of enhancing cytotoxic activity of antibody drug

containing about 50% the lactoferrin hydrolyzed peptide.

[Industrial Applicability]

Because the active ingredient of the drug for cancer therapy used in the present invention can be obtained from a comparatively inexpensive raw material such as cow's milk, it can be produced in a large scale. Furthermore, by enhancing cytotoxic activity of complement and antibody drug with the drug of the present invention, target tumor cells can be efficiently killed or injured. Furthermore, it also has an effect of recovering cytotoxic activity of complement and antibody drug for tumor cells having resistance to the antibody drug. That is, combined use of the drug of the present invention meets the request for continuously performing a therapy with an antibody drug even for a patient having recurrence of a malignant tumor having resistance to the antibody drug and judged that therapeutic treatment with the antibody drug is difficult. This evidently provides a novel guidepost for overcoming antibody drug resistance in cancer therapy.

[Brief description of the drawing]

Fig. 1 shows effect of a lactoferrin hydrolysate mixture and a partial peptide of lactoferrin of the present invention on cytotoxic activity of rituximab against Raji cells. The symbol "\*" denotes  $t < 0.001$  relative to Medium (culture broth).

[Name of document] Abstract

[Summary]

[Problem]

An object of the present invention is to provide a highly safe drug which causes no adverse reaction and has an action of enhancing cytotoxic activity of the antibody drugs in the antibody therapies of cancer, in particular, drug-resistant cancer.

[Means to solve the problem]

A drug that comprises a lactoferrin hydrolysate mixture or peptide of lactoferrin that can be obtained by hydrolyzing lactoferrin with a hydrolytic enzyme and has an action of enhancing cytotoxic activity of an antibody drug in an antibody therapy of cancer.

[Selected drawing] None

[Sequence listing]

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<130> P-C40144

<160> 3

<170> PatentIn Ver. 3.1

<210> 1

<211> 708

<212> PRT

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| Met | Lys | Leu | Phe | Val | Pro | Ala | Leu | Leu | Ser | Leu | Gly | Ala | Leu | Gly | Leu |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |
| Cys | Leu | Ala | Ala | Pro | Arg | Lys | Asn | Val | Arg | Trp | Cys | Thr | Ile | Ser | Gln |
|     |     |     |     | 20  |     |     |     | 25  |     |     |     |     | 30  |     |     |
| Pro | Glu | Trp | Phe | Lys | Cys | Arg | Arg | Trp | Gln | Trp | Arg | Met | Lys | Lys | Leu |
|     |     |     |     | 35  |     |     |     | 40  |     |     |     | 45  |     |     |     |
| Gly | Ala | Pro | Ser | Ile | Thr | Cys | Val | Arg | Arg | Ala | Phe | Ala | Leu | Glu | Cys |
|     |     |     |     | 50  |     |     |     | 55  |     |     |     | 60  |     |     |     |
| Ile | Arg | Ala | Ile | Ala | Glu | Lys | Lys | Ala | Asp | Ala | Val | Thr | Leu | Asp | Gly |
| 65  |     |     |     |     |     | 70  |     |     |     |     | 75  |     |     |     | 80  |

|   |     |     |     |
|---|-----|-----|-----|
| Gly Met Val Phe Glu Ala Gly Arg Asp Pro Tyr Lys Leu Arg Pro Val |     |     |     |
|   | 85  | 90  | 95  |
| Ala Ala Glu Ile Tyr Gly Thr Lys Glu Ser Pro Gln Thr His Tyr Tyr |     |     |     |
|   | 100 | 105 | 110 |
| Ala Val Ala Val Val Lys Lys Gly Ser Asn Phe Gln Leu Asp Gln Leu |     |     |     |
|   | 115 | 120 | 125 |
| Gln Gly Arg Lys Ser Cys His Thr Gly Leu Gly Arg Ser Ala Gly Trp |     |     |     |
|   | 130 | 135 | 140 |
| Ile Ile Pro Met Gly Ile Leu Arg Pro Tyr Leu Ser Trp Thr Glu Ser |     |     |     |
| 145   | 150 | 155 | 160 |
| Leu Glu Pro Leu Gln Gly Ala Val Ala Lys Phe Phe Ser Ala Ser Cys |     |     |     |
|   | 165 | 170 | 175 |
| Val Pro Cys Ile Asp Arg Gln Ala Tyr Pro Asn Leu Cys Gln Leu Cys |     |     |     |
|   | 180 | 185 | 190 |
| Lys Gly Glu Gly Glu Asn Gln Cys Ala Cys Ser Ser Arg Glu Pro Tyr |     |     |     |
|   | 195 | 200 | 205 |
| Phe Gly Tyr Ser Gly Ala Phe Lys Cys Leu Gln Asp Gly Ala Gly Asp |     |     |     |
|   | 210 | 215 | 220 |
| Val Ala Phe Val Lys Glu Thr Thr Val Phe Glu Asn Leu Pro Glu Lys |     |     |     |
| 225   | 230 | 235 | 240 |
| Ala Asp Arg Asp Gln Tyr Glu Leu Leu Cys Leu Asn Asn Ser Arg Ala |     |     |     |
|   | 245 | 250 | 255 |
| Pro Val Asp Ala Phe Lys Glu Cys His Leu Ala Gln Val Pro Ser His |     |     |     |
|   | 260 | 265 | 270 |
| Ala Val Val Ala Arg Ser Val Asp Gly Lys Glu Asp Leu Ile Trp Lys |     |     |     |
|   | 275 | 280 | 285 |
| Leu Leu Ser Lys Ala Gln Glu Lys Phe Gly Lys Asn Lys Ser Arg Ser |     |     |     |
|   | 290 | 295 | 300 |
| Phe Gln Leu Phe Gly Ser Pro Pro Gly Gln Arg Asp Leu Leu Phe Lys |     |     |     |
| 305   | 310 | 315 | 320 |
| Asp Ser Ala Leu Gly Phe Leu Arg Ile Pro Ser Lys Val Asp Ser Ala |     |     |     |
|   | 325 | 330 | 335 |
| Leu Tyr Leu Gly Ser Arg Tyr Leu Thr Thr Leu Lys Asn Leu Arg Glu |     |     |     |

|   |     |     |
|---|-----|-----|
| 340   | 345 | 350 |
| Thr Ala Glu Glu Val Lys Ala Arg Tyr Thr Arg Val Val Trp Cys Ala |     |     |
| 355   | 360 | 365 |
| Val Gly Pro Glu Glu Gln Lys Lys Cys Gln Gln Trp Ser Gln Gln Ser |     |     |
| 370   | 375 | 380 |
| Gly Gln Asn Val Thr Cys Ala Thr Ala Ser Thr Thr Asp Asp Cys Ile |     |     |
| 385   | 390 | 395 |
| Val Leu Val Leu Lys Gly Glu Ala Asp Ala Leu Asn Leu Asp Gly Gly |     |     |
| 405   | 410 | 415 |
| Tyr Ile Tyr Thr Ala Gly Lys Cys Gly Leu Val Pro Val Leu Ala Glu |     |     |
| 420   | 425 | 430 |
| Asn Arg Lys Ser Ser Lys His Ser Ser Leu Asp Cys Val Leu Arg Pro |     |     |
| 435   | 440 | 445 |
| Thr Glu Gly Tyr Leu Ala Val Ala Val Val Lys Lys Ala Asn Glu Gly |     |     |
| 450   | 455 | 460 |
| Leu Thr Trp Asn Ser Leu Lys Asp Lys Lys Ser Cys His Thr Ala Val |     |     |
| 465   | 470 | 475 |
| Asp Arg Thr Ala Gly Trp Asn Ile Pro Met Gly Leu Ile Val Asn Gln |     |     |
| 485   | 490 | 495 |
| Thr Gly Ser Cys Ala Phe Asp Glu Phe Phe Ser Gln Ser Cys Ala Pro |     |     |
| 500   | 505 | 510 |
| Gly Ala Asp Pro Lys Ser Arg Leu Cys Ala Leu Cys Ala Gly Asp Asp |     |     |
| 515   | 520 | 525 |
| Gln Gly Leu Asp Lys Cys Val Pro Asn Ser Lys Glu Lys Tyr Tyr Gly |     |     |
| 530   | 535 | 540 |
| Tyr Thr Gly Ala Phe Arg Cys Leu Ala Glu Asp Val Gly Asp Val Ala |     |     |
| 545   | 550 | 555 |
| Phe Val Lys Asn Asp Thr Val Trp Glu Asn Thr Asn Gly Glu Ser Thr |     |     |
| 565   | 570 | 575 |
| Ala Asp Trp Ala Lys Asn Leu Asn Arg Glu Asp Phe Arg Leu Leu Cys |     |     |
| 580   | 585 | 590 |
| Leu Asp Gly Thr Arg Lys Pro Val Thr Glu Ala Gln Ser Cys His Leu |     |     |
| 595   | 600 | 605 |

|     |     |     |     |
|-----|-----|-----|-----|
| 1   | 5   | 10  | 15  |
| Ser | Ile | Thr | Cys |
| Val | Arg | Arg | Ala |
| Phe | Ala |     |     |
| 20  | 25  |     |     |



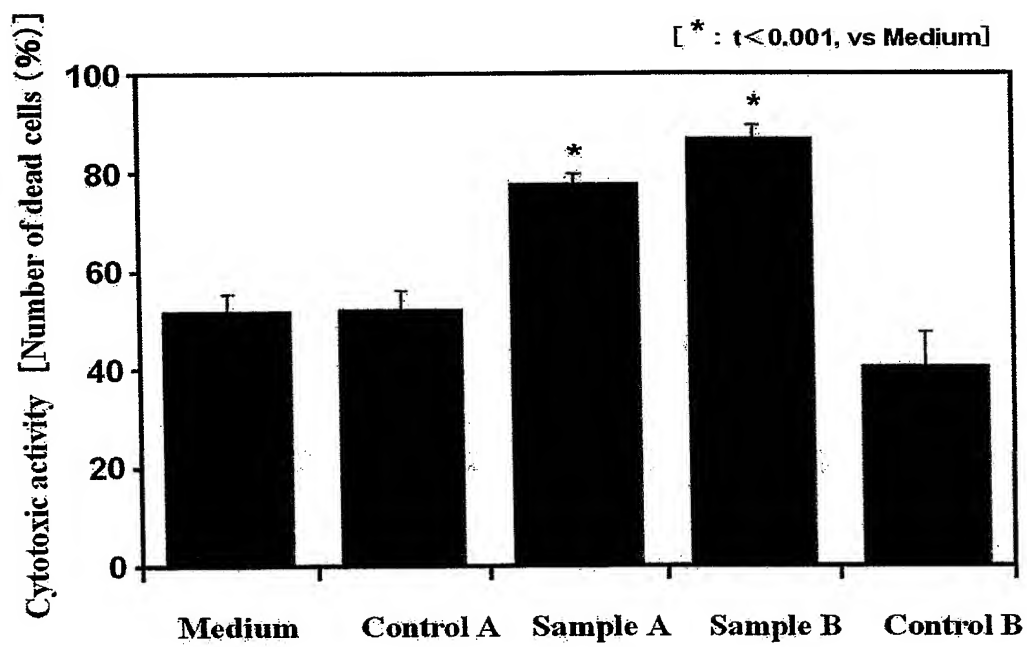


Fig. 1